Novel *in situ* evaluation of the role minerals play in the development of the hard-to-cook (HTC) defect of cowpeas and its effect on the *in vitro* mineral bioaccessibility

Johanita Kruger[a], Roya Minnis-Ndimba[b], Christopher Mtshali[b], Amanda Minnaar[a]

[a] Department of Food Science and Institute for Food, Nutrition and Well-being, University of Pretoria, Private Bag X20, Hatfield 0028, South Africa

[b]Materials Research Department, iThemba LABS, National Research Foundation (NRF), P O Box 722, Somerset West 7129, South Africa.

*Corresponding author: johanita.kruger@up.ac.za, Tel: + 27 12 420-3238, Fax: +27 12 420 2839

Highlights

- We visualised *in situ* mineral distribution in cowpea with PIXE.
- We evaluated the effect of the hard-to-cook (HTC) phenomenon on mineral distribution.
- HTC defect increased Ca and Mg contents in the cell wall-middle lamella of parenchyma cells.
- Results confirm phytate–phytase–mineral hypothesis as a probable mechanisms of HTC defect.

Abstract

Cowpea is a nutritionally important drought-resistant legume in Sub-Saharan Africa. It is, however, underutilised, in part due to the hard-to-cook (HTC) defect caused by adverse storage conditions resulting in seeds not softening during cooking. This study introduced a novel evaluation of the potential role that minerals play in the development of the HTC defect. The mineral distribution in the cotyledons of normal and HTC cowpeas were analysed by Proton Induced X-ray Emission (PIXE) spectrometry. The phytate, tannin and total phenolic contents were analysed together with *in vitro* mineral bioaccessibility. In HTC cowpeas, Ca and Mg were more concentrated in the cell wall-middle lamella area of the parenchyma cells. This, together with the reduction in phytate content, confirmed

the ‗phytase-phytate-mineral' hypothesis as a mechanism for development of the HTC defect. Despite the phytate reduction in stored cowpeas, the HTC defect decreased the bioaccessibility of Ca, Fe and Zn in cowpeas.

Keywords: cowpea; hard-to-cook; HTC; Proton Induced X-ray Emission spectrometry, PIXE; *in situ,* calcium; magnesium; iron; zinc; phosphorus; phytate; mineral bioaccessibility

1 Introduction

Cowpea (*Vigna unguiculata*) is a nutritionally important, protein-rich legume in Asia, Africa and Latin America where it is grown over 10 million ha of land (Nedumaran *et al*., 2013). Its consumption in sub-Saharan Africa has increased at an average rate of 3.2% per year, up to 2009 where on average, 4.54 kg/person/year was consumed. In sub-Saharan Africa there are many individuals suffering from protein energy malnutrition (PEM) and mineral deficiencies (Shrimpton & Rokx, 2013), often due to consumption of monotonous cereal based diets (Hunt, 2003). Cowpea is especially important in these communities as it has a high protein content and is rich in the essential amino acid lysine, which complements the low lysine amino acid composition of cereals (Phillips et *al.,* 2003). Cowpea is also a good source of minerals such as Fe, Zn, Ca and Mg (Sandberg, 2002).

Despite its impressive nutritional characteristics, cowpea, is widely underutilised, in part due to the hard-to-cook (HTC) defect which is caused by adverse storage conditions, resulting in the seeds not softening during cooking (Phillips *et al*., 2003). For short term storage (1-9 months) of legumes the temperature and % relative humidity (RH) should not exceed 30^0C and 50% RH or 20^0C and 60% RH; for intermediate storage (9-18 months) the ranges are 30^0C, 40% RH; or 20^0C and 50% RH or 10^0C and 60% RH (FAO, s.a.). Storage at temperatures and RH higher than prescribed above, is associated with the development of the HTC defect in cowpeas (Hentges *et al*., 1991).

Multiple possible mechanisms have been proposed for the development of the HTC defect (as described by Galiotou-Panayotou *et al.,* 2008; Garcia et *al.,* 1998). However, there is general agreement that there is no single cause for the development of the HTC defect, but rather that many of the proposed mechanisms work together (Liu, & Bourne, 1995; Reyes-Moreno *et al.*, 1993). Still, the most widely accepted proposed mechanism has been the ‗phytase-phytate-pectin' hypothesis (Galiotou-Panayotou *et al.,* 2008; Phillips et *al.,* 2003). This hypothesis states that during storage, high temperatures and high RH conditions are favourable conditions for the activity of intrinsic phytase, the enzyme which dephosphorylates phytate (*inositol* hexa-phosphate). As phytate is dephosphorylated, its ability to chelate divalent minerals such as Ca, Fe, Zn and Mg decreases (Feil, 2001). The divalent minerals are released from mineral-phytate complexes and are then able to move to the cell wall-middle lamella of the parenchyma cells. Here the soluble pectin complexed with monovalent minerals, replaces these, for the divalent minerals (Mattson, 1946). When the carboxyl groups of the pectin bind to divalent minerals, especially Ca, insoluble pectates are formed (Phillips et *al.,* 2003), which act to prevent cell separation during cooking. This results in seeds which do not soften during the cooking process and therefore increase energy requirements and decrease consumer acceptability (Reyes-Moreno *et al.*, 1993).

A challenge in assessing the role of minerals in the ‗phytase-phytate-pectin' hypothesis is that it is difficult to assess the distribution and movement of minerals within the cowpea seed. Normal analytical techniques such as ion coupled plasma (ICP) and atomic absorption spectrometry (AAS) are completely destructive and only provide the total mineral contents of the seeds or larger morphological parts of the seed (when dissected). Proton induced X-ray emission (PIXE) spectrometry is a non-destructive assay and provides quantitative elemental maps displaying the distribution of each mineral *in situ* (Ryan, 2011). While it has recently been used to evaluate the *in situ* mineral distribution in the seed coat, germ and cotyledon of the common bean (Cvitanich *et al*., 2011), it has never been used to evaluate mineral distribution in cowpeas. A limitation of the assay however is that it is not readily available and there are high costs involved compared to conventional methods.

The primary objective of this study was to determine if PIXE could evaluate the role minerals play in the development of the HTC defect. Another objective was to evaluate the effect of the HTC defect on the mineral bioaccessibility of cowpeas in relation to anti-nutrient levels and the mineral contents/distribution. To the knowledge of the authors, no *in situ* evaluation has been done on the movement of the minerals described in the ‗phytase-phytate-mineral' hypothesis. Also, the authors could find no published research on the effect of the HTC defect on the mineral availabilities (*in vitro* and *in vivo)* in cowpea.

2 Materials and methods

2.1 Sample preparation

This study was to serve as a proof of concept for the use of PIXE in evaluating the role of minerals in the development of the HTC defect in legumes. Agrigold cowpea type (Agricol, Potchefstroom, South Africa) with gold colour and wrinkled seed coat was, as it has been found to be highly susceptible to the HTC defect (Yuosuf, 2013). The HTC defect was induced by incubating the seeds at 42ºC and 67% RH for 21 days. Two sets of seeds were placed in a single layer on a net surface over a saturated solution of KCl, for 21 days (Shomer *et al.*, 1990). The position of the two layers was alternated once during the incubation period. The control normal cowpea was stored in a refrigerator at 4ºC.

2.2 Instrumentation and Analytical methods

2.2.1 Cooking times

The cooking times of normal and HTC cowpea seeds were determined using the Mattson Bean Cooker (49.7 g rods). For each test sample, 25 cowpea seeds were positioned in the perforations of the cooker and placed in an aluminium pot with 1.5 L deionised water and boiled. The cooking time was recorded as the moment when 80% of the pins (20 pins) had fallen through the softened seeds (Mwangwela *et al.,* 2006).

2.2.2 Phenolic characteristics

Total phenols were determined using a modified Folin Ciocalteu method (Kaluza *et al*., 1980). Tannin content was determined by the modified Vanillin HCl assay (Price et al., 1978). Reagent blanks that corrected for the colour of the flour extracts were included.

2.2.3 Phytate content

This was determined through anion exchange chromatography, indirect quantitative analysis, measuring the organic phytate-P (inositol-1 to 6- phosphate) (Frubeck et al., 1995). The resin used was Dowex 1; anion-exchange resin-AG 1 x 4, 4% cross-linkage, chloride form, 100-200 mesh (Sigma, Johannesburg, South Africa).

2.2.4 Seed dissection

Seeds (10 whole seeds) of both the normal and HTC cowpea were manually dissected and the seed coat and hilum‘s mineral contents measured. Each morphological part from the 10 seeds were weighed together and used to calculate the % of the total weight of the seed. This was repeated three times.

2.2.5 Mineral content

Mineral analyses of whole flour and dissected seed parts were performed by Ion Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) (SpectroAcros, SPECTRO Analytical Instruments GmbH, Kleve, Germany).

2.2.6 Mineral distribution

The analysis of mineral distribution in the cowpeas were performed using nuclear microprobe at the Materials Research Department of iThemba LABS, South Africa. The facility is equipped with 6 MV single-ended Van de Graaf accelerator and Oxford magnetic quadrupole triplets for beam focusing. Whole kernels of normal and HTC cowpea were coated with a commercial resin (Epofix^{TM} Struers), cut transversely and thinly coated in carbon. A 3x3 µm focused proton beam of 3 MeV and 1.5 MeV energies with approximately 100 pA current was raster scanned over selected areas of the cotyledon (0.5-1.5 mm^2). Scanned areas were typically analysed in a square pattern of up to 128 x 128 pixels, with a dwell time of 10

ms/pixel. PIXE spectra were recorded using a Si (Li) X-ray detector (30 mm2 effective area, working distance of 24 mm) positioned at a take-off angle of 135^{o}. The effective energy resolution of the PIXE system (for the Mn Kα line) was 159 eV. The range of X-rays was set between 1 and 40 keV. Selected areas were measured using two different X-rays absorbers interposed between the sample and PIXE detector. The 125 um Be absorber was used for measurements with 3.0 MeV protons while a 25 µm Be absorber was used for measurement with 1.5 MeV protons. Data were recorded in event-by-event mode using data acquisition system using CAMAC and VME bus modules and XSYS software running on a VAX-4000 computer. The whole system linked to a PC running control system software for stage stepper motors, scanning coils and beam on demand deflecting coils. The PIXE count rate was kept below 1000 counts/s to circumvent pulse pile-up and to achieve satisfactory counting statistics. Samples were irradiated with a total charge between 0.5 and 4 µC. The accumulated PIXE spectra were analysed using GeoPIXE II software (Ryan, 2001). Elemental mapping was performed using Dynamic Analysis method which uses K, L, or M X-ray lines to generate elemental images. A thick sample description was used analysis and the sample matrix was assumed to be comparable to cellulose. Concentration of elements was reported quantitatively in mg/kg.

2.2.7 Scanning Electron Microscopy

The complementary half of the of the cut and carbon coated seeds, analysed by PIXE, were examined using Scanning Electron Microscope (SEM) as described by Taylor et *al.* (2009) with a Jeol JSM-840 SEM (Tokyo, Japan).

2.2.8 Mineral bioaccessibility

Mineral bioaccessibilities were determined according to the dialysis method developed by Luten *et al.* (1996). The mineral contents of the dialysate were analysed by ICP-OES as described. Results are presented as the percentage of the mineral in the dialysate to the total mineral content. Pepsin (P-7000), pancreatin (P-1750), and bile extract (B-8631) were from Sigma. Dialysis tubing used was Spectra/Por 7 (Ø = 20.4 mm) with a molecular weight cut-off (MWCO) of 10 kDa (G.I.C. Scientific, Johannesburg South Africa).

2.2.9 Statistical analyses

All data were analysed by single factor ANOVA (significance level p≤0.05) using Statistica 10 (Statsoft, Tulsa, Oklahoma).

.

3 Results and Discussion

3.1 Effect of the HTC defect on the physical and chemical characteristics of cowpeas

The cooking time of cowpeas stored under adverse conditions more than doubled compared to the cowpeas stored under refrigerated conditions (Table 1). This indicated that the accelerated storage conditions were successful in inducing the HTC defect.

Table 1: Cooking times, phytate, total phenolics, and condensed tannin contents of normal and HTC Agrigold cowpea

	Cooking time (min)	Phytate contents (mg/100 mg)	Total phenolics (mg Catechin Equivalents/100 mg sample)	Condensed Tannins (mg Catechin Equivalents/100 mg sample)
Normal	97 (10)[a]	0.81 (0.06)[b][49%]	0.28 (0.02)[a]	0.35 (0.05)[b]
HTC	218 (74)[b]	0.55 (0.03)[a][38%]	0.25 (0.02)[a]	0.25 (0.04)[a]

() – Values in parentheses are 1SD of 4 analyses

[abc]- values in the same column with different superscripts differ significantly (p≤0.05)

[] – Value in square bracket is the phytate bound phosphorus as a percentage of the total phosphorus (Table 2)

Furthermore, induction of the HTC defect resulted in a significant (p≤0.05) phytate reduction of 32%. In this study, the cowpea was not stored for a long time period (21 days), compared to other studies where cowpea had been stored under adverse conditions for 18 (Liu et *al*., 1993) to 24 months (Hentges *et al*., 1991).

Hentges *et al*. (1991) found that storage of cowpea in adverse conditions for 24 months nearly dephytinised the cowpea. The phytate reduction in the current study was similar to that observed in HTC common beans (28%) and lentils (31%), which were stored for similar periods (Galiotou-panayotou *et al*., 2008). The phytate reduction was probably due to the intrinsic phytase activity of the cowpea, which has been found to be around 140 U/kg (Azeke *et al.,* 2011). The phytate reduction, in this study, agreed with the _phytase-phytate-pectin' hypothesis, which states that induction of the HTC defect results in phytate being dephosphorylated or in effect the content reduced (Reyes-Moreno *et al.*, 1993).

The HTC defect did not result in a significant ($p>0.05$) reduction in total phenolics (Table 1). While the reduction in condensed tannins was significant ($p\leq0.05$), the tannin content was low and the small reduction of 0.1 mg Catechin Equivalents (CE)/100 mg was not of practical importance.

There were no substantial differences between the mineral contents of the whole normal and HTC cowpeas (Table 2). The Ca, Mg and P contents of the cowpea were within previously reported ranges, while that of Fe and Zn were slightly higher and lower, respectively (Boukar *et al.,* 2011).

Considering the phytate and mineral contents together; phytate is highly charged with 6 phosphate molecules capable of chelating positively charged molecules (Feil, 2001). The normal cowpea in this study, contained approximately 132 moles/kg of divalent minerals (Sum of Ca, Mg, Fe & Zn in Table 2), compared to the 12.3 moles/kg phytate (Table 1). The HTC defect resulted in the phytate being reduced by 4 moles/kg, which indicated that up to 24 moles/kg (18%) of the divalent minerals, could have been released. As most of the phytate in legumes are complexed with Mg and Ca (Urbano *et al*., 2000), it could be argued that these were the bulk of the minerals released from these complexes.

There were substantial microstructural differences between the normal (Figure 1 A & C) and HTC (Figure 1 B & D) cowpea as imaged by SEM. There was no clear definition of the parenchyma cells in the normal cowpea (Figure 1-A). The HTC cowpea, however, had highly distinguishable white polygonal parenchyma cell patterns (Figure 1-B, encircled). At larger magnification of the HTC cowpea (Figure

Table 2: Mineral contents of whole and dissected seeds and mineral bioaccessibilites of normal and HTC Agrigold cowpea

		Ca	Mg	Fe	Zn	P
Mineral contents of whole cowpea (mg/kg)	**Normal**	660 (17)[a]	2767 (41)[b]	109 (6)[a]	14 (1.3)[a]	4643 (190)[b]
	HTC	654 (19)[a]	2398 (39)[a][**-13**]	100 (5)[a]	15 (1.3)[a]	4103 (12)[a][**-12**]
Mineral contents of hilum (mg/kg)	**Normal**	2610 (699)[a]	2126 (556)[a]	115 (16)[a]	17.6 (2.2)[a]	ND
	HTC	3138 (874)[a]	2350 (389)[a]	145 (54)[a]	18.2 (3.8)[a]	ND
Mineral contents of seed coat (mg/kg)	**Normal**	5223 (563)[a]	3459 (328)[a]	308 (44)[a]	10.2 (2.5)[a]	812 (92)[a]
	HTC	4902 (249)[a]	3822 (475)[a]	351 (46)[a]	9.6 (1.0)[a]	1522 (144)[b][**47**]
Mineral bioaccessibility (%)	**Normal**	9.6 (2.1)[a]	18 (0.7)[a]	0.99 (0.18)[b]	12 (0.48)[b]	48 (6.9)[a]
	HTC	5.1 (2.9)[b][**-47**]	17 (1.3)[a]	0.61 (0.06)[a][**-38**]	8.2 (0.48)[a][**-32**]	52 (4.4)[a]

ND – Nothing detected limit

[] - % change compared to the mineral content of the normal cowpea

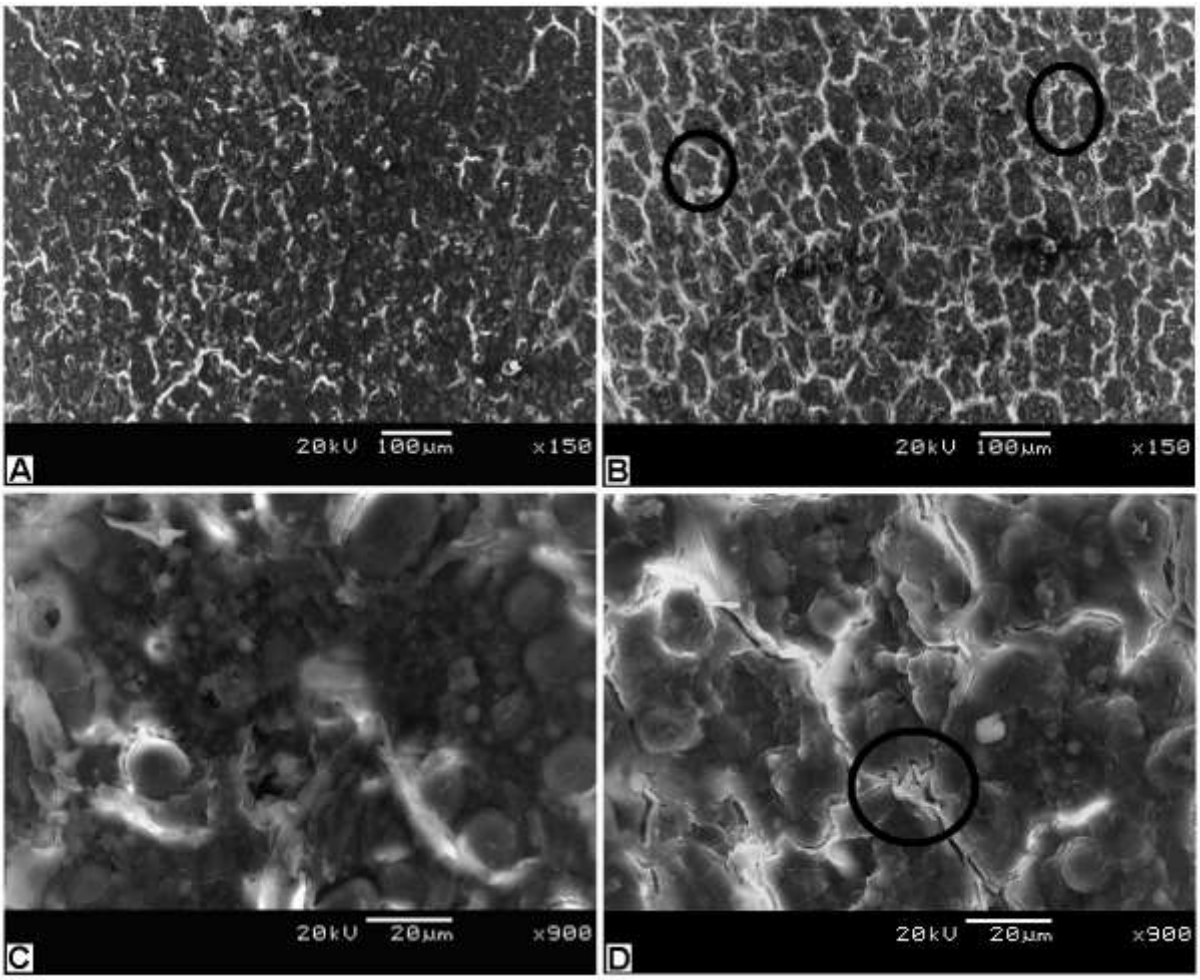


Figure 1: Scanning Electron Microscope(SEM) image of the cotyledon of transversely cut normal (A & C) and HTC (B & D) Agrigold cowpea. Encircled area (B) is an individual parenchyma cell and encircled area (D) is the thickened cell wall-middle lamella

1-D), the thickened cell walls of the parenchyma cells were clearly visible. Although not the focus of the study the thickened cell walls in HTC beans has previously been described (Garcia *et al*., 1998). It is in these visible cell wall-middle lamella areas of the parenchyma cells where pectin is located (Waldron *et al.*, 2003).

3.2 Effect of the HTC defect on the mineral distribution within the cotyledon and seed coat of cowpea

In this study, elemental mapping from scanned areas were used to evaluate the micro mineral distribution in and around the parenchyma cells of the cowpea cotyledon (Figure 2). The ICP-OES analyses of the morphological parts were used to observe the macro mineral distribution in the seed coats (Table 2). This was

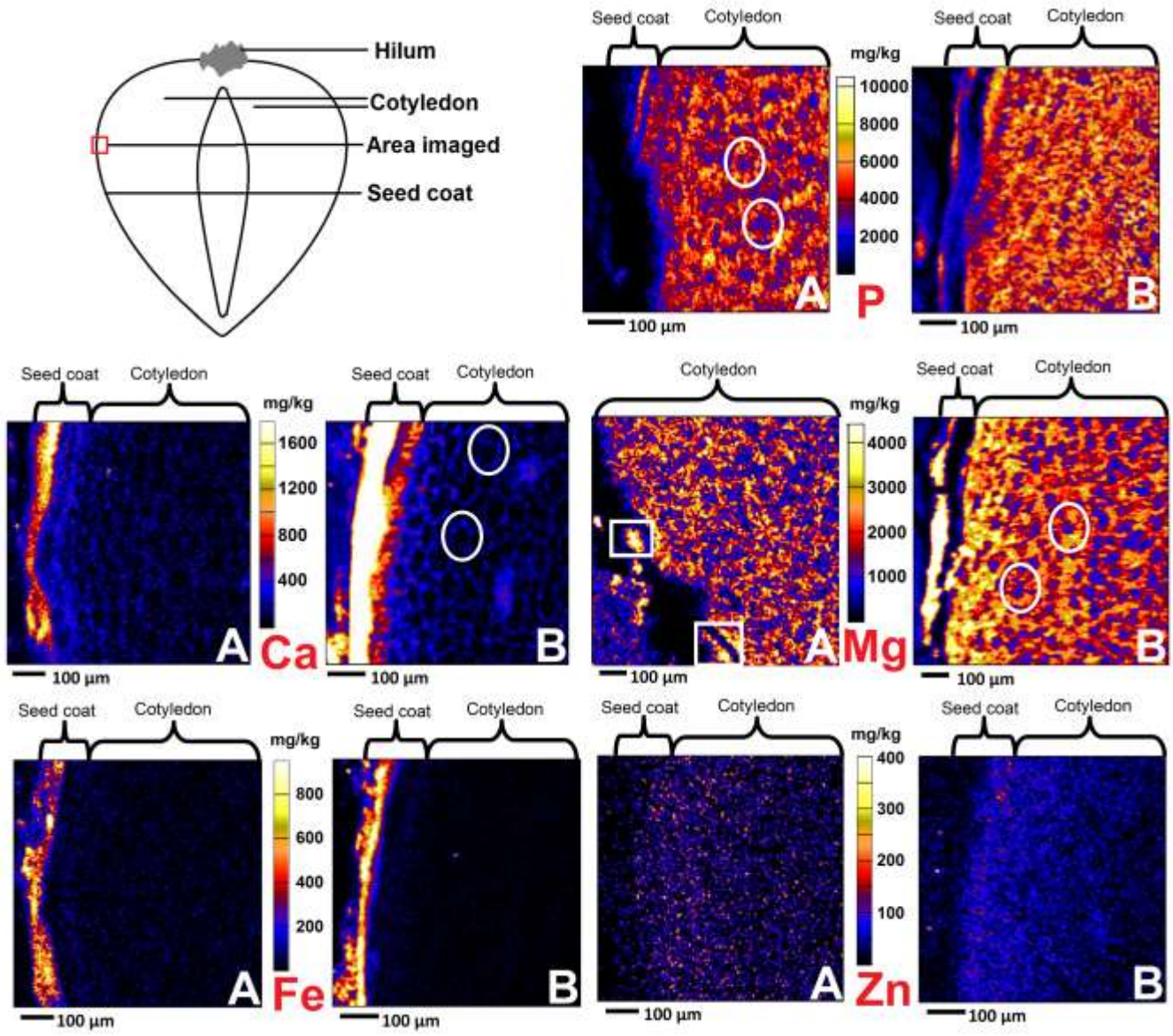


Figure 2: Proton Induced X-ray Emission (PIXE) elemental maps of the P, Ca, Mg, Fe and Zn distribution in the cotyledon of transversely cut normal (A) and Hard-to-cook (HTC) (B) Agrigold cowpeas. Encircled areas are polygonal-like distribution of minerals similar to the parenchyma cells observed in Figure 1B. The blocks (Mg A) surrounds the disintegrated seed coat

because of considerable differences in the mineral concentrations between, normal and HTC cowpeas, seed coats and cotyledons (e.g. Ca and Fe) (Figure 2). As a result, the maximum of the elemental concentration scales (perpendicular Z-direction scale of the contour plots) on the elemental maps were adjusted to ensure that the mineral distribution in the cotyledons of the normal and HTC cowpeas were comparable and clear, despite overexposure or underexposure of the seed coats. This adjustment together with natural variation of the mineral

distribution in a seed coat (Kruger *et al*., 2014), resulted in the PIXE data on the mineral distribution in the seed coats not being used in this study.

Elemental maps revealed polygonal distribution patterns in the cotyledons of the cowpeas formed by P in the normal cowpea (A), and Ca and Mg in the HTC cowpea (B) (Figure 2-encircled). While the patterns of P, Ca and Mg were not equally district, they were very similar in shape and distribution compared to the parenchyma cells observed in the SEM image (Figure 1B-encircled).

In the cotyledon of the normal cowpea, the P distribution, formed distinguishable, low P concentration areas (≈2000 mg/kg) surrounded by higher concentrated areas (≈4000-9000 mg/kg) (Figure 2-encircled). In other words, P concentration appeared to be higher in the cell wall-middle lamella area (when size and distribution of pattern was compared with Figure 1B). Phytate, the main storage form of P in grains can store up to 85% as phytate-P (Feil, 2001). The observation of P distribution, in this study, in normal cowpeas was in direct contrast to previous reports. In legumes it has been found that phytate was located in the protein bodies within the parenchyma cells (Lott & Buttrose, 1978; Urbano *et al.,* 2000). However, only 49% and 38% of the P in the normal and HTC cowpea respectively, were found to be phytate-P (Table 1), which was similar to that previously reported for cowpea (36% phytate-P) (Lott *et al.,* 2000). No information could be found on the distribution of inorganic P (not phytate-P) in the parenchyma cells of cowpea or other legumes.

In the cotyledon of the HTC cowpea, the calcium formed distinct, polygonal patterns of low Ca concentration (≈0-100 mg/kg), surrounded by higher Ca concentrated edges (≈300 mg/kg) (Figure 2-encircled) (Suppl figures 1.1 & 1.2). Similar patterns of Ca distribution have previously been observed in the cotyledon of common beans (Cvitanich *et al.,* 2011). The Mg in the cotyledon of the HTC cowpea also formed low Mg concentration (≈100 mg/kg) areas, surrounded by high Mg concentrated areas (≈2000-4000 mg/kg) (Figure 2-encircled). The Mg and Ca distributions in the cotyledons of the normal cowpea were more evenly distributed compared to the HTC cowpea.

While the pattern of the Mg distribution observed in the HTC cowpea, appeared to be similar to that of the Ca distribution, it was not as clearly defined (Figure 2-encircled). This could be due to the fact that the Mg content (≈2500 mg/kg) of cowpea was so much higher than the Ca content (≈650 mg/kg) (Boukar *et al.,* 2011; Table 2). Even in the cotyledon, the approximate Mg concentration range was 1000-4000 mg/kg, which was more than 10 times higher than the Ca concentration range (Figure 2). It was however clear that in the HTC cowpea, the cell wall-middle lamella of the parenchyma cells (identified in Figure 1 and Section 3.1), had a higher concentration of Ca and Mg, compared to the normal cowpea.

Despite the higher concentration of Mg in the cotyledon of the cowpea (Table 2), the Ca distribution is crucial in the HTC defect evaluation (Linehan & Hughes, 1969). Linehan and Hughes (1969) used a mineral chelator to demineralise a pectin solution, after which different cations (Na^+, K^+, Mg^{2+} and Ca^{2+}) were added and their effect on the intercellular adhesion measured. The Na, K, Mg and Ca cations increased the intercellular adhesion by 150, 190, 280 and 520%, respectively. This indicated the superior effect of Ca on the pectin solubility and cell wall stiffening compared to the other cations. This was because cations, other than Ca, do not form the "egg box" structure when complexed with pectin (Rose, 2003). Furthermore, it was also found to be due to the fact that the ionic radius of Ca, 0.1 nm is large enough to coordinate with oxygen atoms as in many sugars and also because of flexibility with regard to the directions of its coordinate bonds (Taylor, 1992). This was confirmed when out of 13 ions (including mono, di and trivalent ions) soaking in $CaCl_2$ induced the most severe hard-to-cook state in cowpea (Lui *et al.,* 1992).

There was no distinct pattern of distribution observed in any of the Fe or Zn elemental maps of the cotyledons (Figure 2). There was also no substantial difference between the Fe and Zn distributions of the normal and HTC cowpea. This observation, together with the low levels of Fe and Zn compared to Ca and Mg, indicated that after the storage period of 21 days, neither Zn nor Fe played a substantial role in the development of the HTC defect.

The mineral distribution of Ca and Mg in the HTC cowpea cotyledons agreed with the "phytase-phytate-pectin" hypothesis. In this hypothesis, it was proposed that

divalent minerals (Ca and Mg) after being released from the phytate-mineral complex were free to relocate to the cell wall-middle lamella area (Mattson, 1946; Phillips et *al.,* 2003). Furthermore, once relocated, Ca and Mg probably bound to the carboxyl groups of the pectin, resulting in the increased concentrations in these areas.

While the reduction in phytate could have released substantial amounts of divalent minerals (see section 2.2), it does not appear to have been solely responsible for the movement and relocation of minerals. The mineral distributions observed were probably also because of non-phytate bound minerals (e.g. P in the cell wall-middle lamella area). It has to be mentioned that the movement of minerals, observed in this study, would probably not be possible without the degradation of the cell membranes as described by Richardson & Stanley (1991).

The HTC defect did not result in changes in the mineral contents of the seed coats of cowpeas, except for that of P. The P content of the HTC seed coat was increased in the HTC cowpea. This could be because of the release of P during the dephosphorylation of phytate and movement together with other inorganic P due to the degradation of the cell membranes (Richardson & Stanley, 1991).

3.3 Effect of the HTC defect on the mineral bioaccessibility in the cowpea

Surprisingly, Ca, Fe and Zn bioaccessibilities of the HTC cowpeas were reduced (Table 2), despite the reduction in phytate content (Table 1). It is possible that the insoluble Ca-pectin complexes, which have been proposed to form during development of the HTC defect (Mattson, 1946; Phillips et *al.,* 2003), could be the reason for the reduction in Ca bioaccessibility.

Regarding Fe and Zn, it is possible that the phytate reduction was not large enough to result in increased bioaccessibilities. In fact, the phytate reduction reduced the phytate:Fe and phytate:Zn molar ratios from 6.3 and 57 to 4.7 and 36, respectively. These reduction were not close to the proposed critical levels above which Fe (>1 - Hunt 2003) and Zn (>10-14 - Saha *et al.*, 1994) availabilities are seriously impaired. This, however, did not explain the reduction in iron and zinc bioaccessibilities. Martínez-Meyer *et al*. (2012) found that the HTC defect

increased the iron and zinc bioaccessibilities of some beans, while it also decreased the iron bioaccessibility of one cultivar and did not affect the iron or zinc bioaccessibility of other cultivars. There are other factors such as oxalates which can affect mineral bioavailability in legumes (Sandberg, 2002), and It is possible that there might be another mechanism which inhibited the mineral bioaccessibilities, brought on/developed by the HTC defect.

4 Conclusions

PIXE analysis shows considerable potential in evaluating the role minerals play in the development of the HTC defect in cowpeas. The HTC defect, when induced over a relatively short period of time, has a substantial effect on the P, Ca and Mg distribution in the cotyledons of cowpea. Ca and Mg move and concentrate around the cell wall-middle lamella where it probably binds to pectin, insolubilising it. The HTC defect negatively affects the Ca, Fe and Zn bioaccessibilities. More research, including for e.g. investigation into different storage conditions and cultivars is necessary to confirm the effect of the HTC defect on the mineral bioaccessibility and possible mechanisms of inhibition in cowpea. Also, as this was an initial study more research is needed into *in situ* analysis of the role minerals play in the development of the HTC defect of cowpea. In both cases, more cowpea varieties and legume cultivars stored under adverse conditions for longer periods should be analysed.

5 Acknowledgements

The authors would like to thank the staff at the Materials Research Department, in particular Prof C. Pineda-Vargas for his assistance during the PIXE measurements and the evaluation of data. Also Shaakira Yousuf from UP for HTC sample preparation and some analyses.

6 References

Azeke, M. A., Elsanhoty, R. M., Egielewa, S. J., & Eigbogbo, M. U. (2011). The effect of germination on the phytase activity, phytate and total phosphorus

contents of some Nigerian-grown grain legumes. *Journal of the Science of Food and Agriculture, 91,* 75-79.

Boukar, O., Massawe, F., Muranaka, S., Franco, J., Maziya-Dixon, B., Singh, B., & Fatokun, C. (2011). Evaluation of cowpea germplasm lines for protein and mineral concentrations in grains. *Plant Genetic Resources*, *9*, 515-522.

Cvitanich, C., Przybyłowicz, W. J., Mesjasz-Przybyłowicz, J., Blair, M. W., Astudillo, C., Orłowska, E., & Stougaard, J. (2011). Micro-PIXE investigation of bean seeds to assist micronutrient biofortification. *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms*, *269,* 2297-2302.

Food and Agricultural Organisation. (s.a.) Seed management. http://www.fao.org/ag/agp/AGPC/doc/publicat/FAOBUL4/FAOBUL4/B410.htm (September, 2014)

Feil, B. (2001). Phytic acid. *Journal of New Seeds*, *3,* 1-35.

Frubeck, G., Alonso, R., Marzo, F., & Santidrian, S. (1995). A modified method for the indirect quantitative analysis of phytate in foodstuffs. *Analytical Biochemistry*, *225,* 206-212.

Galiotou-Panayotou, M., Kyriakidis, N. B., & Margaris, I. (2008). Phytase–phytate–pectin hypothesis and quality of legumes cooked in calcium solutions. *Journal of the Science of Food and Agriculture*, *88*, 355-361.

Garcia, E., Filisetti, T. M., Udaeta, J. E., & Lajolo, F. M. (1998). Hard-to-cook beans (Phaseolus vulgaris): involvement of phenolic compounds and pectates. *Journal of Agricultural and Food Chemistry*, *46*, 2110-2116.

Hentges, D. L., Weaver, C. M., & Nielsen, S. S. (1991). Changes of selected physical and chemical components in the development of the hard-to-cook bhean defect. *Journal of Food Science*, *56*, 436-442.

Hunt, J. R. (2003). Bioavailability of iron, zinc, and other trace minerals from vegetarian diets. *The American Journal of Clinical Nutrition*, *78,* 633S-639S.

Kaluza, W. Z., McGrath, R. M., Robberts C., & Schroeder, H.H. (1980). Separation of phenolics of sorghum bicolor (L.) Moench grain. *Journal of Agricultural and Food Chemistry, 28,* 1191-1196.

Kruger, J., Pineda-Vargas, C. A., Minnis-Ndimba, R., & Taylor, J. R. N. (2014). Visualisation of the distribution of minerals in red non-tannin finger millet using PIXE microanalysis. *Cereal Science,* In press, available online: http://www.sciencedirect.com/science/article/pii/S0733521014000824 (May, 2014).

Linehan, D. J., & Hughes, J. C. (1969). Texture of cooked potato: III.—Intercellular adhesion of chemically treated tuber sections. *Journal of the Science of Food and Agriculture*, *20*, 119-123.

Liu, K., & Bourne, M. C. (1995). Cellular, biological, and physicochemical basis for the hard-to-cook defect in legume seeds. *Critical Reviews in Food Science & Nutrition*, *35*(4), 263-298.

Liu, K., Phillips, R. D., Hung, Y. C., Shewfelt, R. L., & Mcwatters, K. A. Y. (1992). Hard-to-Cook Defect in Cowpeas: Storage-Induced and Treatment-Induced Development. *Journal of food science*, *57*(5), 1155-1160.

Liu, K., Phillips, R. D., & McWatters, K. H. (1993). Mechanism of pectin changes during soaking and heating as related to hard-to-cook defect in cowpeas. *Journal of Agricultural and Food Chemistry*, *41*, 1476-1480.

Lott, J. N. A., & Buttrose, M. S. (1978). Location of reserves of mineral element in seed protein bodies: macadamia, walnut, and hazel nut. *Canadian Journal of Botany*, 56, 2072-2082

Lott, J. N., Ockenden, I., Raboy, V., & Batten, G. D. (2000). Phytic acid and phosphorus in crop seeds and fruits: a global estimate. *Seed Science Research, 10,* 11-34.

Luten, J., Crews, H., Flynn, A., Van Dael, P., Kastenmayer, P, Hurrell, R., Deelstra, H., Shen, L., Fairweather-Tait, S., Hickson, K., Farre, R., Schlemmer, U.

& Frøhlich, W., (1996). Interlaboratory trial on the determination of the in vitro iron dialysability from food. *Journal of the Science of Food and Agriculture*, *72*, 415-424.

Martínez Meyer, M. R., Rojas, A., Santanen, A., & Stoddard, F. L. (2013). Content of zinc, iron and their absorption inhibitors in Nicaraguan common beans (*Phaseolus vulgaris* L.). *Food chemistry, 136,* 87-93

Mattson, S. (1948).The cookability of yellow peas. *Soil Science*, *66*, 77.

Mwangela, A. M., Waniska, R. D., & Minnaar, A. (2006). Hydrothermal treatments of two cowpea (VignaunguiculataL. Walp) varieties: effect of micronisation on physicochemical and structural characteristics. *Journal of the Science of Food and Agriculture, 86,* 35-45.

Nedumaran, S., Abinaya, P., Shraavya, B., Rao, P. P., & Bantilan, M. C. S. (2013). Grain legumes production, consumption and trade trends in developing countries-an assessment and synthesis. *Socioeconomics Discussion Paper Series nr 3*, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT).

Phillips, R. D., McWatters, K. H., Chinnan, M. S., Hung, Y. C., Beuchat, L. R., Sefa-Dedeh, S., Sakyi-Dawson, S., Ngoddy, P., Nnanyelugo, D., Enwere, J., Komey, S. M., Liu, K., Mensa-Wilmot, Y., Nnanna, I. A., Okeke, C., Prinyawiwatkul, W., & Saalia, F. K. (2003). Utilization of cowpeas for human food. *Field Crops Research*, *82*, 193-213.

Price M. L., Van Scoyoc S., & Butler L. G. (1978). A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. *Journal of Agricultural and Food Chemistry, 26,* 1214-1218.

Reyes-Moreno, C., Paredes-López, O., & Gonzalez, E. (1993). Hard-to-cook phenomenon in common beans - A review. *Critical Reviews in Food Science & Nutrition*, *33*, 227-286

Rose, J. K. (Ed.). (2003). *The plant cell wall* (Vol. 8). CRC Press.

Ryan, C. G. (2001). Developments in dynamic analysis for quantitative PIXE true elemental imaging. *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms*, *181*, 170-179.

Ryan, C. G. (2011). PIXE and the nuclear microprobe: Tools for quantitative imaging of complex natural materials. *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms*,*269*(20), 2151-2162.

Saha, P. R., Weaver, C. M., & Mason, A. C. (1994). Mineral bioavailability in rats from intrinsically labelled whole wheat flour of various phytate levels. *Journal of Agricultural and Food Chemistry, 42*, 2531-2535.

Sandberg, A. S. (2002). Bioavailability of minerals in legumes. *British Journal of Nutrition*, *88*, 281-285.

Shomer, I., Paster, N., Lindner, P., & Vasiliver, R. (1990). The role of cell wall structure in the hard-to-cook phenomenon in beans (*Phaseolus vulgaris* L.). *Food Structure, 9,* 139-149.

Shrimpton, R., & Rokx, C. (2012). Double Burden of Malnutrition – A Review of Global Evidence. HNP Discussion paper, World bank, http://www-wds.worldbank.org/external/default/WDSContentServer/WDSP/IB/2013/07/15/000445729_20130715150604/Rendered/PDF/795250WP0Doubl00Box037737900PUBLIC0.pdf (viewed May, 2014).

Taylor, J., Taylor, J. R., Belton, P. S., & Minnaar, A. (2009). Preparation of free-standing films from kafirin protein microparticles: mechanism of formation and functional properties. *Journal of Agricultural and Food Chemistry*, *57*, 6729-6735.

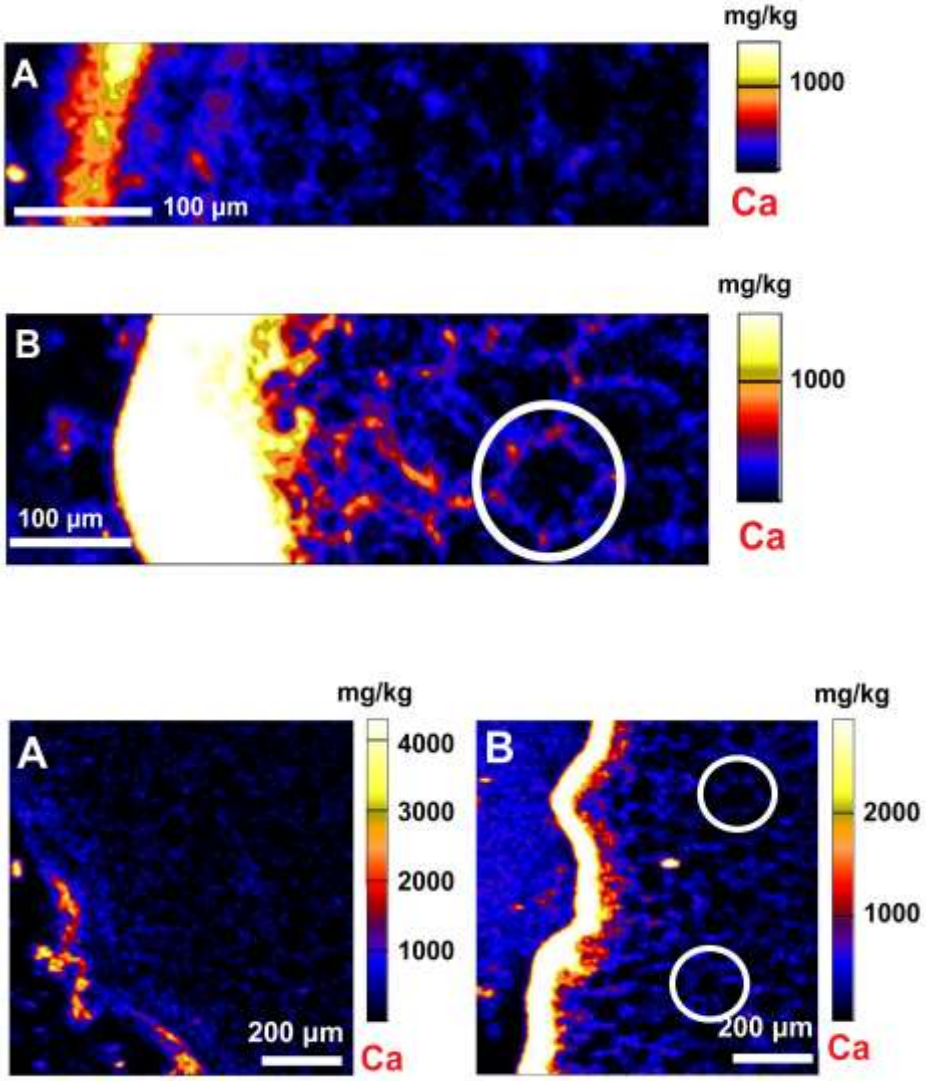
Taylor, S. (1992). *The chemistry and technology of pectin*. R. H. Walter (Ed.). Academic Press.

Urbano, G., Lopez-Jurado, M., Aranda, P., Vidal-Valverde, C., Tenorio, E., & Porres, J. (2000). The role of phytic acid in legumes: antinutrient or beneficial function?. *Journal of physiology and biochemistry*, *56*, 283-294.

Waldron, K. W., Parker, M. L., & Smith, A. C. (2003). Plant cell walls and food quality. *Comprehensive Reviews in Food Science and Food Safety*, *2*, 128-146.

Yuosuf, S. 2013. Improvement of the nutritional quality of hard-to-cook cowpeas using a hydrothermal treatment. Honours degree Project, Department of Food science, University of Pretoria, South Africa.

Appendix A: Supplementary data



Supplementary figure 1.1 & 1.2: Proton Induced X-ray Emission (PIXE) elemental maps of the Ca distribution in the cotyledon of transversely cut normal (A) and Hard-to-cook (HTC) (B) Agrigold cowpeas. Encircled areas are polygonal-like distribution of minerals similar to the parenchyma cells observed in Figure 1B.